Pyrroline-5-Carboxylate Reductase in Lactating Bovine Mammary Glands[1]

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ABSTRACT

The occurrence and subcellular distribution of pyrroline-5-carboxylate reductase have been studied in lactating bovine mammary glands. The enzyme appears to have only a cursory association with the mitochondrial fraction, because significant amounts of the enzyme are found in other membrane-containing fractions and in the cytosol. Polyamines stimulate the enzyme in vitro, supporting the mediation of cursory attachment to membrane fractions by these compounds. The enzyme is selective for NADPH but can utilize NADH as well. Long-chain coenzyme A derivatives, which are generated during lipid metabolism, almost completely inhibit this enzyme, which is responsible for the synthesis of a portion of the proline needed for casein production. Overall, the enzyme concentration in the gland correlates well with a role in the conversion of an intermediate, L-Δ^1-pyrroline-5-carboxylate, into proline, an important amino acid for the mammary secretory process, especially casein synthesis.

(**Key words**: milk secretion, enzymes, proline)

Abbreviation key: **CoA** = coenzyme A, K_m = Michaelis constant, **OAT** = ornithine-δ-aminotransferase, **P5C** = pyrroline-5-carboxylate.

INTRODUCTION

Ruminants, such as the dairy cow, have the capability of converting properly supplemented forage into proteins of nutritionally superior quality. One intriguing aspect of protein production is the uptake of amino acids by lactating mammary epithelial cells. Mepham (10) described three classes of amino acids with regard to mammary uptake in relation to protein production: class 1, those adequately taken up; class 2, those not adequately taken up; and class 3, those taken up in excess of protein production. An interesting relationship appears to exist between proline (a class 2 amino acid) and ornithine and arginine (class 3 amino acids). To account for the high amounts of proline required for casein synthesis, enzymes related to the urea cycle were postulated to participate in the metabolic conversion of ornithine and arginine to proline (5, 13, 18). Recently, Basch et al. (2) studied one key enzyme in this process, ornithine-δ-aminotransferase (**OAT**), which was shown to occur primarily in the mitochondria of the lactating bovine mammary gland. The OAT converts ornithine to pyrroline-5-carboxylate (**P5C**). In turn, P5C can be reduced to proline by the action of P5C reductase (L-proline:$NADP^+$ 5-oxidoreductase; EC 1.5.1.2), which has not been reported to occur in the lactating bovine mammary gland. This paper reports on the occurrence of P5C reductase in the lactating bovine mammary gland and compares the properties of the mammary enzyme with similar enzymes in other tissues (11, 14, 15).

MATERIALS AND METHODS

Materials

The DL-Δ^1-P5C acid, β-NADPH (reduced form), acetophenone, *o*-aminobenzaldehyde, palmitoyl coenzyme A (**CoA**), stearoyl-CoA, proline, putrescine, adenosine-5′-triphosphate, β-NADPH (oxidized), *p*-hydroxymercuribenzoic acid, spermine, and spermidine were purchased from Sigma Chemical Co. (St. Louis, MO). Mercuric chloride was obtained from Alfa Products (Ward Hill, MA), and potassium dihydrogen phosphate was purchased from Mallinckrodt Specialty Chemicals Co. (Paris, KY). Absolute ethyl alcohol was obtained from Warner-Graham Co. (Cockeysville, MD), and toluene (Photrex®) was purchased from J. T. Baker Incorporated (Phillipsburg, NJ). Trichloroacetic acid was obtained from Fisher Scientific Co. (Pittsburgh, PA).

Whole mammary glands from three midlactation, multiparous Holstein cows of known good health and productivity (about 20 kg/d of milk) were obtained from the USDA herd (Beltsville, MD). The glands were collected at slaughter, trimmed to remove adi-

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[1]Mention of brand name or firm names does not constitute an endorsement by the USDA over others of a similar nature.

pose tissue, and sectioned into 500-g pieces, which were frozen on dry ice and stored at –80°C until used. Tissue fractionation was carried out essentially according to the method described by Basch et al. (1) by first mincing the mammary glands and then suspending them in a homogenization buffer. This buffer was composed of 37.5 m*M* Tris-maleate (adjusted to pH 7.0 to preserve P5C reductase), 1 m*M* EDTA, 5 m*M* $MgCl_2$, 0.1 m*M* dithiothreitol, and 0.25 *M* sucrose. The glands were homogenized and passed through three layers of cheesecloth to remove connective tissue. Subcellular fractions (nuclei, mitochondria, microsomes, and cytosol) were isolated by differential centrifugation as previously described (1). All pellets were resuspended in homogenization buffer and recentrifuged at the appropriate speeds. Pellets were then redispersed in a volume equal to 10% of that used for homogenization and stored at –80°C.

The substrate, DL-Δ^1-P5C acid, was prepared from 2,4-dinitrophenyl hydrazone of glutamic-γ-semialdehyde according to the procedure of Mezl and Knox (12).

Enzyme Assays

The NADPH:P5C reductase activity was measured at 25°C by monitoring the decrease in absorbance at 340 nm according to the method of Shiono et al. (15). The standard reaction mixture in disposable 1-cm semimicropolystyrene cuvettes contained 500 μl of 100 m*M* KH_2PO_4 (pH 6.05), 30 μl of 10 m*M* NADPH, 200 μl of 10 m*M* P5C, 230 μl of distilled water, and 40 μl of the bovine mammary gland fraction that contained the enzyme. Change in absorbance at 340 nm was monitored using a Beckman DU 650 spectrophotometer (Beckman Instruments, Fullerton, CA). This instrument was capable of automatically subtracting the blank rate to obtain the corrected rate. When rate results were calculated, the blank solution reading was subtracted from the corresponding sample reading at each time point for every sample. The rate was then calculated using linear regression to determine the best fit of a straight line to the data obtained during the assay. The reaction was started by the addition of the enzyme. A sample without substrate contained all components except P5C, which was replaced by distilled water and served as the control. When NADH was used as the cofactor, the higher concentrations that were required for activity exceeded the linearity of the spectrophotometer (absorbance >2.0). Therefore, these assays were conducted in standard 1-cm cells; solid quartz spacers (0.896 cm) were added to reduce the path length and lower the absorbance.

Assays for NADPH:cytochrome *c* reductase and succinate dehydrogenase activities were conducted as previously described (1).

Citrate synthase activity was measured spectrophotometrically according to the procedure for bovine mammary gland as described by Basch et al. (2). Readings were recorded at 412 nm with a Beckman DU 650 spectrophotometer.

Substrate Assay

The concentration of P5C was determined by reaction with *o*-aminobenzaldehyde according to the method of Mezl and Knox (12). The reaction mixture in microcentrifuge tubes contained 450 μl of P5C sample (adjusted to pH 6.0 to 6.1 with dilute KOH), 500 μl of 10% trichloroacetic acid, and 50 μl of 0.1 *M* *o*-aminobenzaldehyde in 40% (vol/vol) ethanol. For samples without substrates, 0.25 *M* hydrochloric acid was used in place of P5C. The assays were incubated at 25°C for 25 min, and the mixture then was centrifuged at 10,800 × *g* for 15 min at 25°C with a TOMY microcentrifuge (Peninsula Laboratories Inc., Belmont, CA). The supernatant was carefully transferred into a 1-cm cuvette, and the optical density at 440 nm was measured with a Beckman DU 650 spectrophotometer.

Protein Assay

Protein was determined using the Pierce bicinchoninic acid protein assay reagent (Pierce Chemical Co., Rockford, IL) using the room temperature protocol. Bovine serum albumin was the standard.

Data Analysis

Nonlinear regression analyses of standard Michaelis curves and variance of parameters with concentration were carried out using the program Abacus, which is based on the Gauss-Newton iterative method (6). Choices between fits of models and statistical methods of analysis of the nonlinear fits were as described by Farrell et al. (7). For plots of velocity against concentration of substrate, [S], which deviated from standard patterns, data were fitted with equations originally derived from Wyman's theory of thermodynamic linkage and subsequently adapted for use in enzyme kinetics experiments (7). The simplest expression used in these experiments is an expansion of the general Michaelis constant (K_m) expression:

$$V_{obs} = \frac{V_{max}\,[S]^n}{K_m^n + [S]^n} \qquad [1]$$

TABLE 1. Subcellular distribution of pyrroline-5-carboxylate reductase in preparations from lactating mammary glands.[1]

Bovine fraction	Specific activity (nmol/min per protein)		Yield (%)		Ratio to homogenate	Enzyme marker ratio[2]
	$\bar{X}$	SD	$\bar{X}$	SD		
Homogenate	16.9	8.0	. . .	. . .	. . .	1.0
Nuclear	24.1	11.0	3	3	1.4	. . .
Mitochondrial	24.0	6.0	16	6	1.4	5.6[3]
Microsomal	12.0	5.9	6	1	0.7	5.3[4]
Cytosolic	15.8	6.2	56	18	0.9	. . .

[1]Means and standard deviations from three preparations from three cows. Three assays for each preparation.

[2]Mean values for three preparations.

[3]Succinate dehydrogenase assay; ratio of specific activity in mitochondria to homogenate.

[4]Assay for NADPH:cytochrome *c* reductase; ratio of specific activity in microsomes to homogenate.

where V_{obs} = observed V and V_{max} = maximum V. For enzyme kinetics in general, n = 1; for cooperative mechanisms, n > 1.

RESULTS

Reaction Conditions

Preliminary studies on lactating bovine mammary glands showed P5C reductase activity in both mitochondrial and cytosolic fractions. The accuracy of the enzymatic assay was tested on both fractions. The P5C reductase assay was linear with time up to 8 min for mammary fractions with and without substrate. The rate for mammary samples without substrate was subtracted from the rate for the complete reaction mixture to account for NADPH oxidase activity. The response was linear with midranges of 40 and 20 μl for cytosolic protein fraction and mitochondrial preparation, respectively. This amount represents 400 μg of cytosolic and 240 μg of mitochondrial protein. Undiluted bovine mammary gland fractions worked best with the assay, but, if diluted fractions were needed, homogenization buffer was recommended for use to preserve enzyme activity. Thus, the assay (15) appeared to work quite well with mammary fractions.

After the conditions for linearity with time and enzyme were established, both bovine mammary cytosolic and mitochondrial fractions were assayed to determine their optimal pH. The ideal pH maxima are between pH 6 and 7, and the pH maxima was 6.05 (Figure 1) for both fractions. The previous literature (14) indicated that the preservation of P5C enzyme activity was highly dependent on buffer and temperature. The maximum stability at –20°C, as reported in the literature (14), was at most 2 mo. In this study, the P5C enzyme was stabilized up to 1 yr by storage at –80°C in the homogenization buffer.

Subcellular Distribution

The subcellular distribution of P5C reductase in the bovine mammary tissue is given in Table 1. Three subcellular preparations were made from frozen mammary tissues of three cows. The table shows that the specific activity was the greatest for both mitochondrial and nuclear fractions, and also the specific activity ratio of the subcellular fraction to

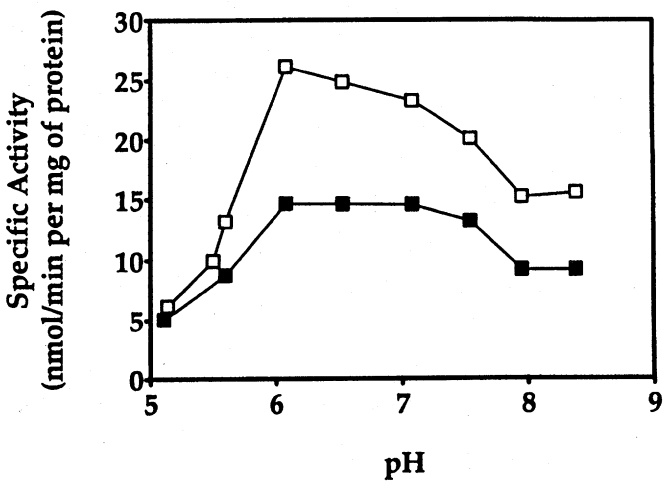

Figure 1. pH activity curves for pyrroline-5-carboxylate reductase of mitochondrial (□) and cytosolic (■) fractions of the bovine mammary gland.

TABLE 2. Subcellular distribution of citrate synthase in preparations of lactating mammary glands.[1]

Bovine fraction	Specific activity		Yield		Ratio to homogenate
	— (nmol/min per protein) —		— (%) —		
	$\bar{X}$	SD	$\bar{X}$	SD	
Homogenate	97	21	. . .	. . .	. . .
Mitochondrial	22	73	27	10	2.3
Cytosolic	100	18	69	8	1.0

[1]Means from three preparations from three cows; three assays for each preparation.

homogenate was greatest for the same fractions. Because OAT is a mitochondrial enzyme (2), P5C reductase for maximal efficiency ought to be associated with the mitochondrial fraction as well. Despite the fact that the specific activity and its ratio of the fraction to homogenate were the same in the mitochondrial and nuclear fractions, the yield of P5C reductase was five times greater in mitochondria than in the nuclei. However, the yield of P5C reductase in cytosol was the highest among all subcellular fractions. Marker enzyme ratios (Table 1) for the mitochondrial and microsomal fractions were about 5:1 and were well within values reported for the bovine mammary gland (1, 2, 8).

The 56% yield of P5C reductase in the cytosol (Table 1) could reflect mitochondrial breakage during freezing and thawing of the tissue or mitochondrial disruption during mincing and homogenization of the tissue. To assess the degree of breakage for these preparations, citrate synthase, a marker for mitochondrial integrity, was assayed in three fractions. Table 2 gives the subcellular distribution of citrate synthase. These results showed that, with homogenization, bovine mammary glands averaged 69% breakage of the mitochondrial fraction under the described buffer conditions. The ratios of mitochondrial fraction to homogenate were 5.6 for succinate dehydrogenase, 2.3 for citrate synthase, and 1.4 for P5C reductase. Thus, P5C enzyme was not as tightly associated with mitochondria as were succinate dehydrogenase and citrate synthase. Hence, activity in the cytosol could be due to mitochondrial breakage or perhaps to the occurrence of a single soluble form of the enzyme that has a cursory association with membrane fractions.

Heat Stability

The thermal stability of the bovine mitochondrial and cytosolic fractions at 37°C was tested against time with the standard assay. The two fractions were very similar and retained 100% of catalytic activity after incubation for 4 h at 37°C. Thus, P5C reductase was very stable in the presence of the Tris-maleate (pH 7.0) buffer. In contrast, heating for 4 min at 100°C completely abolished P5C reductase activity in both fractions.

K_m

Kinetic analyses of P5C reductase with varying concentrations of P5C and NADPH resulted in typical curves (Figures 2 and 3). Nonlinear fits to velocity versus substrate concentrations were calculated as previously described. In the presence of 0.3 m*M* NADPH as a cofactor, the mammary enzyme gave K_m for P5C of 0.37 ± 0.05 m*M* (Figure 2) for both the mitochondrial and cytosolic fractions (mean of three experiments each). In the presence of 2 m*M* P5C as substrate, K_m were 0.073 and 0.049 m*M* (Figure 3) for NADPH with the mitochondrial and cytosolic fractions, respectively. The plots of velocity against substrate concentration for both the mitochondrial and cytosolic fractions exhibited distinct cooperativity with n = 2 for NADPH (Equation [1]).

For purified P5C reductase (11), NADPH can be replaced by NADH. This change of cofactor increased maximum velocity four times for mammary cytosol. This rate increase was deceptive because the K_m for NADH using mammary cytosol was 0.18 m*M*, which was nearly four times greater than that obtained for NADPH.

Inhibition Studies

The NADPH-dependent activity of P5C reductase could be fully, partially, or not inhibited by long-chain acyl-CoA, nucleotides, amino acids, heavy metals, and polyamine second messengers. Table 3 illustrates the results obtained with selected compounds. Palmitoyl-CoA and stearoyl-CoA are both potent inhibitors, as shown in Table 3. Stearoyl-CoA inhibited the NADPH-dependent activity of P5C reductase in the cytosolic fraction to a greater extent than that in the

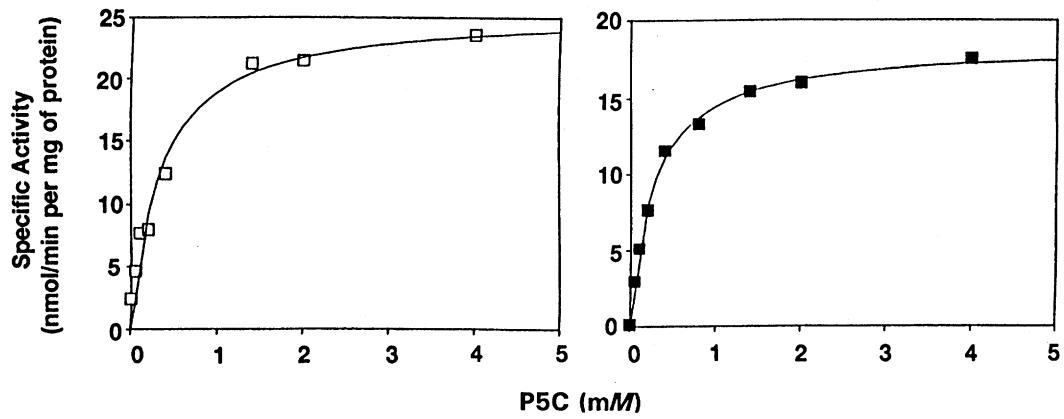

Figure 2. Change in the velocity (specific activity) of reaction of pyrroline-5-carboxylate (P5C) reductase of bovine mammary mitochondrial (□) and cytosolic (■) fractions with increasing concentration of P5C substrate. The concentration of NADPH was constant at 0.3 m*M*.

mitochondrial fraction. Inhibition of enzyme activity was slight or nonexistent with either ATP or $NADP^+$ for both cytosolic and mitochondrial fractions. The P5C reductase activity was markedly inhibited by *p*-hydroxymercuribenzoate, and Hg^{2+} alone was also an effective inhibitor. Partial inhibition of NADPH-dependent P5C reductase activity was observed with high concentrations of proline (5 and 10 m*M*). As shown in Table 3, the enzyme activity in the mitochondrial fraction was inhibited by proline almost two times more than was the cytosolic P5C activity. Interestingly, putrescine, spermine, and spermidine stimulated NADPH-dependent P5C reductase activity up to 36%. These second messenger compounds contained diamine groups, which might have increased activity by binding to the enzyme.

DISCUSSION

Ornithine-δ-aminotransferase is considered to be a mitochondrial matrix enzyme that shuttles carbon skeletons and functions in the production of proline (2, 13, 15, 18) in several tissue types. Studies on the uptake of proline by mammary cells have indicated that insufficient proline is taken up to support casein production by fully lactating cells (5, 10, 13), which indicates a need for proline biosynthesis within the mammary gland (5, 10, 13). One study (13) using ^{14}C-labeled tracers indicated that ornithine could serve as a source of proline. That conclusion was in agreement with results of earlier studies (5, 10), which showed that ornithine is readily taken up by lactating mammary cells. The mechanism for conversion of arginine or ornithine to proline could occur in

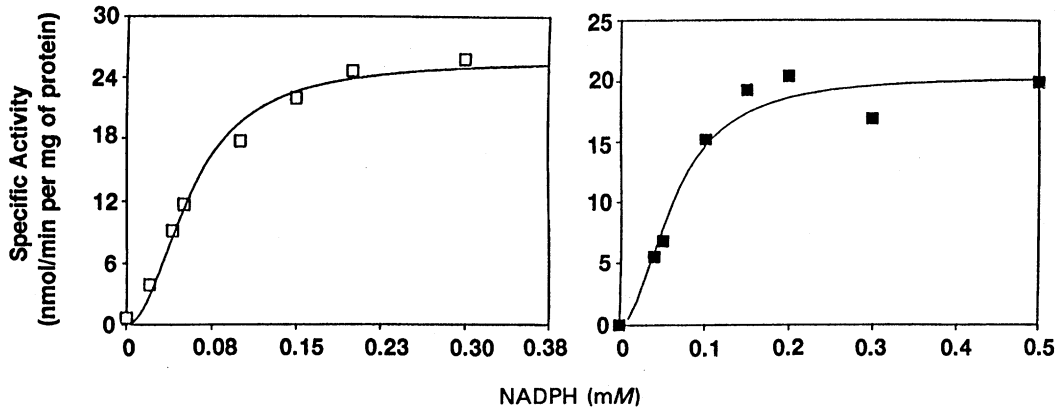

Figure 3. Change in the velocity (specific activity) of reaction of pyrroline-5-carboxylate (P5C) reductase of bovine mammary mitochondrial (□) and cytosolic (■) fractions with increasing concentration of NADPH as the cofactor. The concentration of P5C was constant at 2 m*M*.

TABLE 3. Inhibitory and stimulatory effects of selected compounds on pyrroline-5-carboxylate reductase activity.[1]

Compound	Concentration	Cytosol		Mitochondria	
		Inhibition (%)			
	(m*M*)	$\bar{X}$	SD	$\bar{X}$	SD
Palmitoyl-CoA[2]	0.3	91	2.5	87	3.4
Stearoyl-CoA	0.3	92	0.8	50	15.7
ATP	1	3	5.9	17	15.3
$NADP^+$	1	9	5.3	5	14.4
Proline	5	37	20.1	69	16.2
Proline	10	42	20.3	68	12.4
$HgCl_2$	0.01	79	3.9	88	5.5
p-OH Mercuribenzoic acid	0.1	90	2.6	95	0.7
		Activation (%)			
Putrescine	1	5	0.6	7	2.6
Putrescine	5	11	0.5	14	7.3
Spermine	1.2	13	0.7	36	8.2
Spermidine	1.2	10	1.2	15	0.9

[1]Means from three preparations from three cows. Three assays for each preparation.

[2]CoA = Coenzyme A.

two ways (15), but, in either case, OAT and P5C reductase would be important enzymes. The first step in the conversion of ornithine to proline is the action of OAT to produce the δ-amino semialdehyde, which cyclizes to yield L-Δ^1-P5C. This enzyme has recently been shown to occur in lactating tissues (2). The second step is the reduction of P5C to form proline.

Results from our study show that the P5C reductase enzyme is present in the lactating mammary gland. The specific activity in the crude homogenate is 16.9 nmol/min per mg of protein, which is very similar to that of OAT in the homogenate (17.6 nmol/min per mg of protein) (2). In the bovine mammary mitochondrial fraction, the specific activity of P5C reductase is about one-half that of OAT. The yield of enzyme and the ratio of activity in mitochondria to homogenate for P5C reductase are also about one-half those of OAT. In contrast, the data in the microsomal fraction indicated that the specific activity, yield, and ratio to homogenate of P5C reductase were six times greater than those of OAT. Therefore, P5C reductase activity is more evenly distributed in subcellular fractions than is OAT. Apparently, breakage of the mitochondrial fractions of bovine mammary gland was considerable, as was evidenced by parallel occurrence of citrate synthase, P5C reductase, and OAT (2) in the cytosolic fractions, but the ratio of mitochondria to homogenate is nearly two times greater for citrate synthase than for P5C reductase. The data for subcellular distribution of citrate synthase in this report are very similar to those reported by Basch et al. (2). Using the latter enzyme as a marker, in this study, at pH 7.0, the frozen bovine mammary glands averaged 69% breakage of the mitochondrial fraction compared with an average of 64% breakage in the previous study (2). At pH 7.4, with Tris buffer and fresh bovine tissue, Farrell et al. (8) reported 30% mitochondrial breakage. In contrast, at pH 6.7, breakage of mitochondria in fresh rat mammary tissue was 47% (2). Apparently, subtle changes in homogenization buffer can alter the fragility of bovine mitochondria during the vigorous homogenization needed to disrupt mammary connective tissue. There is some support for two forms of P5C reductase, a membrane-associated form and a soluble (cytosolic) form. Evidence for two forms of P5C reductase in the mammary gland arises because the mitochondrial activity is more sensitive to inhibition by proline, less sensitive to stearoyl-CoA inhibition, and has a higher K_m for NADPH. However, second messenger diamines can promote enzyme mobilization and promote binding of enzymes to membrane components (16). The stimulation of P5C reductase by polyamines may be important in this respect; the polyamines increase mitochondrial activity to a greater extent than cytosolic activity (Table 3). Thus, the same cytosolic enzyme bound to membranes may differ in activity from its free form. For the previously mentioned reasons, conclusive evidence for two isozymes is currently absent.

Using the starting assumptions of Waghorn and Baldwin (17), a moderately productive cow with a 21.7-kg udder could easily yield 15 kg/d of milk. Based on a mean casein content of 2.89% (4), yield is 433 g/d or 18.8 mmol/d of casein or roughly 3760 mmol/d of amino acids incorporated into casein pro-

tein. Because casein contains 12.3 mol of proline/100 mol of amino acids (H. M. Farrell and H. J. Dower, 1994, unpublished data), 460 mmol/d of proline are incorporated into casein or 320 μmol/min and 15 nmol/min of proline per g of tissue. This value compares well with the mean value for proline incorporation (21 nmol/min per g of tissue) that was calculated from the overall data of Cant et al. (3). Basch et al. (2) reported that 960 nmol/min per g of tissue of L-Δ^1-P5C, the immediate precursor of proline, could be produced if OAT functioned near maximum velocity. This study showed a potential to produce 610 nmol/min per g of tissue of proline when P5C reductase operated near maximum velocity. These values signify that P5C reductase could convert almost two-thirds of the potential proline precursor produced by OAT.

The K_m values of 0.37 m*M* obtained in this study for P5C are in accord with earlier data for the enzyme P5C reductase from other tissues (11, 14). In the experiments utilizing NADPH, both the cytosolic and mitochondrial enzymes displayed cooperativity (n = 2), and K_m values of 0.049 and 0.073 m*M* are also in accord with previous data (11, 14). The cofactor NADH also may be used by P5C reductase, and the K_m as reported previously (14) is about 0.3 m*M*; for mammary cytosol, K_m was 0.18 m*M*. Such a high K_m generally precludes NADH as a source of reducing equivalents under most physiological conditions (11), especially for the lactating bovine mammary gland in which the NAD^+:NADH ratio is 220:1, and the $NADP^+$:NADPH ratio is 1:25,000 (17).

Product inhibition by proline occurred only at relatively high concentrations and may not be a factor in the mammary gland in vivo. The inhibition by sulfhydryl reagents was in accord with data for this enzyme from other sources (11, 14, 15).

Several dehydrogenases can be inhibited by long-chain acyl-CoA derivatives (9). Palmitoyl-CoA, which arises from the major end product of mammary lipid biosynthesis (palmitate) (3, 17), almost completely inhibits P5C reductase, as does stearoyl-CoA, which arises from a dietary lipid (stearate). The inhibition of this proline (and, hence, protein-generating enzyme) by these lipid metabolites could explain the potential for protein depression with supplemental dietary fat (3). In contrast to long-chain acyl-CoA derivatives, short-chain derivatives have little effect on purified mammary isocitrate dehydrogenase (9). Should the same be true for P5C reductase, short- to medium-chain fats in the diet could possibly depress fat synthesis by inhibiting acetyl-CoA carboxylase (3, 17), but actually could stimulate protein synthesis by having no effect on P5C reductase or isocitrate dehydrogenase.

CONCLUSIONS

Recently, the consumption of full fat dairy products has declined because of consumer interest in reducing fat and cholesterol in the diet. Therefore, increased protein production with a concurrent reduction in fat production is a future goal of milk producers. To accomplish this goal, a better understanding of the mechanisms that control milk protein synthesis and secretion, coupled with an assessment of rate-limiting steps in milk protein production, could pave the way to sites of future intervention to alter the ratio of protein to fat. This study reported on the occurrence of P5C reductase in lactating mammary glands of the cow and provides an indication that this reducing enzyme functions after OAT in the conversion of ornithine into proline. The inhibition of P5C reductase by long-chain acyl-CoA derivatives indicates a possible tie between protein and lipid synthesis in the lactating mammary gland.

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